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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<b>(21) International Application Number:</b> PCT/BR97/00087 <b>(22) International Filing Date:</b> 30 December 1997 (30.12.97)  <b>(71) Applicant (for all designated States except US):</b> UNIVERSIDADE FEDERAL DE MINAS GERAIS [BR/BR]; Avenida Antonio Carlos, 6627, Bairro São Francisco, CEP-31270-901 Belo Horizonte, MG (BR).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> PEREGRINO FERREIRA, Paulo, César [BR/BR]; Apartamento 201, Alameda dos Jacarandás, 23, Bairro São Luiz, CEP-31275-060 Belo Horizonte, MG (BR). GEESSIEN KROON, Erna [BR/BR]; Avenida Xangri-Lá, 75, Braúnas, CEP-31365-640 Belo Horizonte, MG (BR). BERNARDES MARGUTTI PINTO, Maria, Elizabeth [BR/BR]; Rua Monteiro Lobato, 211, Bairro Ouro Preto, CEP-31310-530 Belo Horizonte, MG (BR). ALEIXO, Agdemir, Wáleria [BR/BR]; Rua Salinas, 139, Boa Vista, CEP-35700-155 Sete Lagoas, MG (BR).		<b>(81) Designated States:</b> US, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> A METHOD FOR THE DIAGNOSIS, IDENTIFICATION AND CHARACTERIZATION OF <i>M. TUBERCULOSIS</i> AND OTHER MYCOBACTERIA BY SHIFT MOBILITY ASSAY  <b>(57) Abstract</b> <p>The present invention relates to a method for diagnosis, identification and characterization of <i>M. tuberculosis</i> or any other mycobacteria by using PCR and shift mobility assay (SMA). The method is based both on microheterogeneities observed within the 16S rRNA sequences established for mycobacteria and on nucleotide gaps and mismatches which cause a decrease in the electrophoretic mobility of DNA heteroduplex in polyacrylamide gels. The PCR strategy is based on the use of a specific primers for mycobacteria genera and divergence in sequences found in 16S rRNA coding gene. The difference in nucleotide sequence was enough to identify mycobacteria species, since a remarkable shift between single stranded and homoduplex bands in PAGE were observed among mycobacteria tested, when <i>Mycobacterium tuberculosis</i> was used as standard. The shift of PCR product of bacteria other than mycobacteria was observed above the single stranded band in PAGE, both in standard cultures and clinical specimens. SMA can thus provide a fast and sensitive method for detection and classification of mycobacteria in clinical samples as well as pure culture.</p>		

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**A METHOD FOR THE DIAGNOSIS, IDENTIFICATION AND CHARACTERIZATION OF *M. TUBERCULOSIS* AND OTHER MYCOBACTERIA BY SHIFT MOBILITY ASSAY.**

5 TECHNICAL FIELD OF THE INVENTION

The present invention relates to a method for detect, identify and classify *Mycobacterium tuberculosis* or any other mycobacteria, by using a urea-poliacrylamide gel (UPAGE) to distinguish between heteroduplex and homoduplex shift bands obtained by mixing PCR products derived from 16S rRNA coding gene fragment of mycobacteria.

- 10 The method is based on divergence in sequences found in 16S rRNA to identify mycobacteria species, since a remarkable shift of heteroduplex bands are obtained between single stranded and homoduplex bands in UPAGE.

BACKGROUND OF THE INVENTION

- 15 Tuberculosis (TB) and other mycobacterial illnesses remain the number one cause of infectious diseases related deaths worldwide. Annual increases of 10% per year have been reported (Bloom, B. R., and C. J. Murray. Science 257:1055-1064, 1992 ; Centers for Disease Control. MMWR 40:58, 1991) in part related to the epidemic of HIV. At the same time an increasing number of atypical presentations of AIDS patients with *Mycobacterium tuberculosis*
- 20 (MTB) are also seen, with a vast increase in extra pulmonary diseases (Persing, D.H., J. Clin. Microbiol. 29:1281-1285, 1991). Of even greater concern than the increase in absolute numbers of mycobacterial infections is the emergence of previously rare strains, collectively termed mycobacteria other than tuberculosis (MOTT). Moreover, of particular importance, is the emergence of MTB multi drug-resistant, which causes over 90% mortality in
- 25 immunocompromised hosts (Shankar, P., N. Manjunath, R. Lakshmi, B. Aditi, P. Seth, and Shriniwas. Lancet 335:423, 42. 1990) .

- The conventional diagnosis of tuberculosis based on the gold standard, culture-grown organisms, is extremely sensitive (10-100 organisms per ml present for growth) but is expensive and time-consuming (2-8 weeks), because of the slow doubling rate (Vaneechoutte M., H.
- 30 Beenhouwer, G. Claeys, G. Verschraegen. A. de Rouck, N. paepe, A. Alachouni, and F. Portaels. J. Clin. Microbiol. 31:2061-2065. 1993) of acid-fast bacilli (AFB). Direct staining procedures are rapid but lack sensitivity and specificity since *Legionella* and *Nocardia* are stained

as well (Rogall, T., J. Wolters, T. Flohr, and E.C. Boettger. *Int. J. Syst. Bacteriol.* 40:323-330, 1990).

By overcoming the inherent limitations of stain and culture techniques, molecular methods have great potential for the rapid diagnosis of mycobacteria. The polymerase chain reaction (PCR) was reported (Cousins, D.V., S.D. Wilton, B.R. Francis, and B.L. Gow. *J. Clin. Microbiol.* 30:255-258, 1992) to be useful in the direct diagnosis of tuberculosis infection directly from a variety of clinical specimens (Bocart, D., D. Lecossier, A. Lassence, D. Valeyre, J. P. Battesti, and A.J. Hance. *Am Rev Respir Dis* 145:1142-1148, 1992 ; Brisson-Noël, A., B. Gicquel, D. Lecossier, V. Lévy-Frebault, X. Nassif, and A.J. Hance. *Lancet* ii:1069-1071, 1989) with a diversity of genetic elements used as target templates. The rapid detection of MTB by PCR and the high degree of sensitivity in clinical samples afforded by DNA amplification have been described by various investigators (Cousins, D.V., S.D. Wilton, B.R. Francis, and B.L. Gow. *J. Clin. Microbiol.* 30:255-258, 1992 ; Del Portillo, P., L.A. Murillo, and M.E. Patarroyo. *J. Clin. Microbiol.* 29:2163-2168, 1991 ; Folgueira, L., R. Delgado, E. Palenque, and A.R. Noriega. *J. Clin. Microbiol.* 31:1019-1021, 1993). However, there are a number of difficulties to be overcome before these methods can be used as a routine diagnosis test (Garcia-de-Lomas J. and D. Navarro. *J.* 16:S43-S48, 1997). Nevertheless, PCR analysis of chromosomal DNA is a simple and rapid method for identifying MTB to the species level and in clinical specimens ( Shawar, R.M., F.A.K. El Zaatari, A. Nataraj, and J.E. Clarridge. *J. Clin. Microbiol.* 31:61-65, 1993).

PCR reaction probes and primers for the insertion fragment IS986 or IS6110 (Cave, M.D., K.D. Eisenach, P.F. MacDermot, J. H Bates, and J.T. Crawford. *Mol. Cel. Probes* 5:73-80, 1991 ; Chevrel-Dellagi, D., A. Abderrahman, R. Haltiti, H. Koubaji, B. Gicquel, and K. Dellagi. *J. Clin. Microbiol.* 31:2446-2450, 1993) have been used and many different strategies have been proposed to confirm the identity of amplified DNA products. One common strategy for detecting and speciating microorganisms is specific gene-probe hybridization and PCR targeted to 16S rRNA subunit (Böddinghaus, B., T. Rogall, T. Flohr, H. Blöcker, and E.C. Böttger. *J Clin Microbiol* 28:1751-1759, 1990), which is coded in mycobacteria by multiple gene (10 genes for 16S RNA) copies per cell . The 16S subunit contains sequences that have highly conserved regions virtually identical in all bacteria (Figure 7), interspersed with other sequences found to be genus- and/or species-specific (Victor, T., R. DuToit, and P.D. VanHeiden. *J. Clin. Microbiol.* 30:1514-1517, 1992). Conserved regions can be used to amplify

rRNA from all prokaryotes or from broadly-related groups of bacteria, while the genus and species-specific interspersed sequence domains can be used to construct specific oligonucleotide probes to detect and differentiate resulting PCR products, or to directly probe mycobacteria in clinical samples by hybridization.

5 Most often, PCR products are visualized with ethidium bromide staining on an agarose gel (Figure 1), with final confirmation on Southern blot. Previously, colorimetric PCR hybridization assays have been investigated as an alternative to gel electrophoresis and Southern blot analysis of PCR-amplified DNA. Such gels and Southern blots have proven to be time-consuming and tedious for typical clinical laboratories as well. Solid-phase colorimetric PCR  
10 assays capture denatured amplification products on probes bound to nylon membranes (as in reverse dot blots) (Plikaytis, B.B., K.D. Eisenach, J.T. Crawford, and T.M. Shinnick. *Molec. Cell. Probes* 15:215-9, 1991 ; Sambrook, K.J., Fritsch, E.F., Maniatis, T., In : *Molecular Cloning. A Laboratory Manual* .USA : Cold Spring Harbor Laboratory Press,1989 ) or to microtiter plates (Thierry, D., M.D. Cave , K.D. Eisenach, J.T. Crawford, J.H. Bates, B.  
15 Gicquel, and J.L. Guesdon. *Nucleic Acids Res.* 18:188-189, 1990). However, the sensitivity of these assays suffer due to the tendency of the denatured PCR product strands to reassociate and exclude oligonucleotide probes, and steric interference between the bound oligonucleotides and the solid support which impede hybridization to nucleic acids in solution (Plikaytis, B.B., K.D. Eisenach, J.T. Crawford, and T.M. Shinnick. *Molec. Cell. Probes* 15:215-9, 1991).

20 Other difficulties encountered using existing techniques include variability from sample preparation and detection of PCR products (Nolte, F.S., B. Metchock, J.E. McGowan, A. Edwards, O. Okwumabaa, C. Thurmond, P.S. Mitchell, B. Plikaytis, and T. Shinnick. *J. Clin. Microbiol.* 31:1772-1782, 1993), technically unmanageable steps to enhance detection, false-positive reactions due to the amplification of contaminating DNA and inhibition of DNA  
25 amplification (Noordhoek, G.T., A.H.J. Kolk, G. BJune, D. Catty, J.W. Dale, P.E.M. Fine, P. Godfrey-Fausset, S-N. Cho, T. Shiinick, S.B. Svenson, S. Wilson, and J.D.A. van Embden. *J Clin Pathol.* 48: 810-814, 1995). These difficulties argue for searching more precise, sensitive, reproducible and low cost assays for MTB diagnosis.

We report here a method for detection and identification of mycobacteria using PCR  
30 strategies targeted to mycobacterial 16S rRNA coding gene and the heteroduplex mobility assay previously developed for analysis of HIV (Delwart, E. L., Herring, B., Rodrigo, A. G. and Mullins, J. I. *PCR Meth. and Appl.*, 1995 ; Delwart, E. L., Shepaer, E. G., Louwagie, J,

McCutchan, F. E. Grez, M., Waigmann, H. R., and Mullins, J. I. Science. 262:1257-1261, 1993). This new approach is fast, simple and can produce information not easily obtained when compared with other methods described so far. Thus, combining molecular methods and PAGE we developed a method which resulted in an integrated strategy to achieve sensitive and specific diagnosis of mycobacteria. Combination of these approaches has wide application in detecting microorganisms and in detection of gene products important to clinical genetic and neoplastic disease.

#### BRIEF DESCRIPTION OF THE DRAWINGS

These and other objects, features and many attendant advantages of the invention will be better understood upon a reading of the following detailed description when considered in connection with the accompanying drawings wherein:

Figure 1 shows a photography of ethidium bromide-stained of PCR amplification products with primers F-285 e ZR-244 derived from DNA of patients with *M. tuberculosis* and *E. coli* standard culture. The PCR products (5µl) were fractioned on a 1% gel agarose at 100 volts for 40 min. and showed an expected 360bp fragment. Lane M, PEL phage DNA digest with Hind III ; lane 2, *M. tuberculosis* ; lane 3, no DNA template; lane 4 to 13 show fragments amplified from clinical samples DNA.

Figure 2 shows a photography of a shift mobility assay of 360bp PCR fragment product derived from 16S rRNA coding gene of various bacteria using as standard *M. tuberculosis*. The PCR product fragment of various bacteria were mix (2.5µl v/v) with mycobacteria, denatured (95°C), re-annealed and run in PAGE at 100 volts for 60 min. Lane 1, *Proteus mirabilis* ; lane 2, *Proteus mirabilis* + *M. tuberculosis* ; lane 3, *Proteus mirabilis* + *M. avium* ; lane 4, *Proteus mirabilis* + *M. fortuitum* ; lane 5, *Pseudomonas aeruginosa*; lane 6, *Pseudomonas aeruginosa* + *M. tuberculosis* ; lane 7, *Pseudomonas aeruginosa* + *M. Avium* ; lane 8, *Pseudomonas aeruginosa* + *M. fortuitum* ; lane 9, *Klebsiella* sp ; lane 10, *Klebsiella* + *M. tuberculosis* ; lane 11, *Klebsiella* + *M. avium* ; lane 12 . *Klebsiella* sp + *M. fortuitum*.

Figure 3 shows a photography of a shift mobility assay of 1030pb PCR fragment derived from 16S rRNA coding gen of Mycobacteria. A PCR fragments from *M. tuberculosis* (standard) and various mycobacteria were mixed (2.5µl v/v). After denaturation (95°C). re-annealed reactions were run in PAGE at 200 volts for 60min.. Lane 1, *M. tuberculosis* ; lane 2, *M. tuberculosis* + *M. avium* ; lane 3, *M. avium* ; lane 4, *M.tuberculosis* + *M. fortuitum* ; lane 5, *M.*

*fortuitum* ; lane 6, *M. tuberculosis* + *M. smegmatis*; lane 7, *M. smegmatis*; lane 8, *M. tuberculosis* + *M. kansasii*; lane 9, *M. kansasii*; lane 10, *M. tuberculosis* + *M. scrofulaceum*; lane 11, *M. scrofulaceum*.

Figure 4 shows a photography of effect of *M. fortuitum* derived 16S rRNA 1030pb PCR product concentration in the shift mobility assay detection. A PCR fragments from standard *M. tuberculosis* (1460ng) and *M. fortuitum* were mix (2.5µl v/v) and denatured (95°C). After re-annealed reactions were run in 5% PAGE with 3% of urea in TBE buffer at 200 volts for 60 min.. Lane 1, *M. tuberculosis* ; lane 2, *M. fortuitum* (1500ng); lane 3, *M. tuberculosis* + *M. fortuitum* (1500ng); lane 4, *M. tuberculosis* + *M. fortuitum* (800ng); lane 5, *M. tuberculosis* + *M. fortuitum*. (400ng); lane 6, *M. tuberculosis* + *M. fortuitum*. (200ng).

Figure 5 shows a photography of effect of DNA template concentration in the heteroduplex mobility assay of 1030pb PCR fragment derived from rDNA of *M. tuberculosis* e *M. fortuitum*. A PCR fragments from *M. tuberculosis* (standard) and *M. fortuitum* derived from different DNA template concentration were mixed (2.5µl v/v) denatured (95°C). re-annealed and run in PAGE at 200 volts for 60 min. Lane 1, *M. tuberculosis* (100ng); lane 2, *M. fortuitum*. (100ng); lane 3, *M. tuberculosis* (10ng) + *M. fortuitum*. (10ng); lane 4, *M. tuberculosis* (5ng) + *M. fortuitum* (5ng); lane 5, *M. tuberculosis* (1ng) + *M. fortuitum* (1ng); lane 6, *M. tuberculosis* (0.1ng) + *M. fortuitum* (0.1ng); lane 7, *M. tuberculosis* (0.01ng) + *M. fortuitum*. (0.01ng).

Figure 6 shows the identification of *M. avium* by heteroduplex mobility shift assay in clinical sample of patient suspected of mycobacteria infection. The assay was performed by using *M. tuberculosis* and *M. fortuitum* as a standard. A PCR fragments from *M. tuberculosis* or *M. fortuitum* were mix (2.5ml v/v) to clinical sample #1 and #2 denatured (95°C) re-annealed and run in PAGE at 200 volts for 60 min.. Clinical sample #1 showed in lanes 1 to 4 is a typical profile pathem of *M. tuberculosis* and and clinical sample #2 ( lanes 5 to 8) of *M. avium*. Lane 1, sample clinical #1; lane 2, sample clinical #1 + *M. tuberculosis* ; lane , sample clinical #1 + *M. avium*; lane 4, sample clinical #1 + *M. fortuitum* ; lane 5, sample clinical #2; lane 6, sample clinical #2 + *M. tuberculosis* ; lane , sample clinical #2 + *M. avium* ; lane 8, sample clinical #2 + *M. fortuitum*.

Figure 7 shows a diagram of 16S RNA and regions where nucleotide sequences are different between some mycobacteria species and *E. coli*.



## DETAILED DESCRIPTION OF THE INVENTION

It is, therefore, an object of the present invention to provide a method of identification, classification or diagnosis for *Mycobacterium tuberculosis* or other mycobacteria that uses the Shift Mobility Assay (SMA). The method outlined here allowed identification of mycobacterium species from culture media or clinical samples based on heteroduplexes formed from 16S rRNA coding genes. The method is based on heteroduplexes migration in polyacrylamide gels observed after denaturing and reannealing mixtures of PCR products derived from various sources of 16S rRNA genes using *M. tuberculosis* or *M. bovis* as standard (Figure 1) When two non divergent sequence fragments were mixed two bands were observed in polyacrylamide gel: one in the bottom (homoduplex) and other in the middle of the gel, which corresponds to the single stranded DNA (Figure 2) due to minor differences in primer concentration, heteroduplexes with reduced mobility are detected when annealed DNA has 1-2% divergent nucleotide sequences or when gaps were presented in one relative to a second sequence (Figure 7). Heteroduplexes were found above the single stranded DNA bands (close to the top of the gel) and between the homoduplexes (bottom of the gel) and single stranded bands. The HMA of amplified PCR products from mycobacteria rDNA derived from culture is shown in Fig. 2 and 4. Specific heteroduplexes were formed between species, even when more than two species were presented in the mixture. Heteroduplex formation was also observed by mixing mycobacteria and other species of bacteria (Fig. 3), but these heteroduplexes migrated slower than the single stranded DNA. Faster migrating heteroduplexes with mobilities faster than ssDNA were observed among all mycobacteria species compared (Fig. 4, 5 and 6).

A variety of commercially available primers in the 16S rRNA coding gene region may be used for PCR amplification.

In order that this invention may be better understood the follow examples for illustrative purposes only, are described. The examples illustrate the present invention and are not intended to limit it in spirit or scope.

## EXAMPLE 1

Bacterial cultures. Bacterial strains as *Mycobacterium bovis*, *Mycobacterium avium*; *Mycobacterium scrofulaceum*; *Mycobacterium kansasii*; *Mycobacterium smegmatis*; *Mycobacterium fortuitum* or other than mycobacteria (*Pseudomonas aeruginosa*;

*Staphylococcus* sp.; *E. coli* ; *Klebsiela* sp. e *Proteus mirabilis*) obtained from the Lowenstein's, agar plate or liquid culture media

## EXAMPLE 2

- 5 DNA extraction. DNA was extracted after lysing 0.1 - 1000 µg of solid microorganism from pure culture with 100 - 500µl of lysis buffer solution (TE pH 6.0-8.0, 0.1-2.0% of lysozyme, 0.1-10 % of Tween 80) for 30-60 min. at 15-30<sup>0</sup>C . SDS was then added to 1-10% plus 50-500µg/ml of proteinase K and the test tube kept for 40-120 min. at 37- 55<sup>0</sup>C. From clinical samples, aliquots of fresh sputum were treated with N-acetyl,L-cysteine (0.5-2.0 mg/ml) for 40-10 60 min, at room temperature, followed by treatment with same volume of lysis buffer as described above. DNA was extracted twice with phenol:chloroform: isoamyl alcohol as described by Boddinghaus et al. (Böddinghaus, B., T. Rogall, T. Flohr, H. Blöcker, and E.C. Böttger. J Clin Microbiol 28:1751-1759, 1990) resuspended in deionized H<sub>2</sub>O or any other hydrophylic solvent and storage at -20 to -80<sup>0</sup>C until use. DNA was measured by 15 spectrophotometry .

## EXAMPLE 3

- DNA amplification. DNA was amplified by using three oligonucleotides primers inside 16S rRNA of *M. tuberculosis* were used: two reverse designated primer ZR-244 20 (CCCACTGCTGCCTCCCGTA) located at nucleotides 298 to 317, MYC-264 located at positions 1027 to 1046 (TGCACACAGGCCACAAGGGA) and a forward primer designated F-285 (AGAGTTTGATCCTGGCTCAG) corresponding to position 8 to 28. The resulting PCR product was 1030bp long when primers F-285 and MYC-264 were used and 360bp long for F-285 and ZR-244. PCR reaction containing 0.1-900µg of template DNA as indicated, 2.0-6.0 mM 25 of MgCl<sub>2</sub>, 4-6 pM of each primer in 50-150 mM Trisma (Sigma, SP, Brazil), pH 7.2-8.3, 50 to 200µM of each dNTP (Pharmacia, São Paulo, Brazil), 2.5-5.0 units of Taq DNA polymerase (Promega, Belo Horizonte, MG, Brasil) and the reaction was performed in a final volume of 20-100µl. PCR reactions were carried out in a Perkin Elmer 4800 thermal cycler (Perkin Elmer/Cetus) for 35-40 cycles, using one sec ramp setting time between steps in a cycle of 93-30 96<sup>0</sup>C for 1-3 min., 50-65 <sup>0</sup>C for 1-5 min., 70-72<sup>0</sup>C for 1-5 min. and a 10-20 min. 65-72<sup>0</sup>C extension step was linked to the last cycle. The annealing temperature for primers F-285 and MYC-264 was 45-70<sup>0</sup>C instead of 37-50<sup>0</sup>C used for primers F-285 and ZR-244. Positive

controls were 0.3µg of DNA from *M. bovis* (BCG). Two negative controls were included: one with no DNA template and the other with *E. coli* DNA (0.5-2µg) in the PCR reaction and no amplification products were observed in these reactions. PCR products were evaluated by electrophoresis on 2-6 % agarose gels and stained with ethidium bromide.

5

#### EXAMPLE 4

Shift mobility assay. The shift mobility assay was performed by mixing 2.5-10µl of PCR products derived from standard *M. tuberculosis* PCR product with 2.5-10µl of PCR product from other species or clinical samples plus 1ul of 10X annealing buffer (1-5mM NaCl, 100-10 500mM Tris-Cl pH7.2-8.0 and 20-40 mM EDTA). The mixture was heated 90-96°C for 2-5 min. then cooled to 25-45°C in the thermocycler. Heteroduplexes were electrophoresed on 3-6 % polyacrylamide gels (25-30:0.8-1.0 Acrylamide:Bis) and 3-7% of urea. Gels were run at 100-200 volts when the 1030bp fragment PCR products were used and at 50-100 volts for 360bp fragments. both for 1 hour, 1X TBE buffer in a mini gel (Hoeffer mini-gel SE 200) apparatus. 15 Gels were than fixed and stained with silver (Stain Plus, Bio-Rad, USA).

While the present invention has been described in connection with examples, it will be understood that modifications and variations apparent to those ordinary skill in the art are within the scope of the present invention.

#### WHAT IS CLAIMED IS:

- 20 1. A method for diagnosis, identification and characterization of *M. tuberculosis* or any other mycobacteria by using PCR and shift mobility assay (SMA) in urea-poliacrylamide gel electrophoresis, comprising the following steps : 1) culture; 2) DNA extraction; 3) DNA amplification; 4) Shift mobility assay
2. The method according claim 1, wherein the the step 1 of culture can optional
- 25 3. The method according claim 1, wherein the DNA extraction (2) from culture (1) or clinical samples can be made with phenol/chlorophorm extraction method or any other method
4. The method according claim 1, wherein the DNA amplification can be made by using any primer in the region of 16S rRNA coding gene, or using any other gene than 16S rRNA coding gene
- 30 5. The method according claim 1, wherein the shift mobility assay are made use of urea in poliacylamide gel electrophoresis to detect, visualize or identify by PCR products derived from sequences of 16S ribosomal RNA coding gene and for detection of heteroduplex complex

observed between single strand (middle of migration) and homoduplex complex bands (bottom of the gel) ;

6. The method according claim 1, wherein use of *M. tuberculosis* or any other mycobacteria as standard to identify by shift mobility assay PCR products derived from sequences of 16S ribosomal RNA coding gene or any other gene.
- 5

FIGURE 1

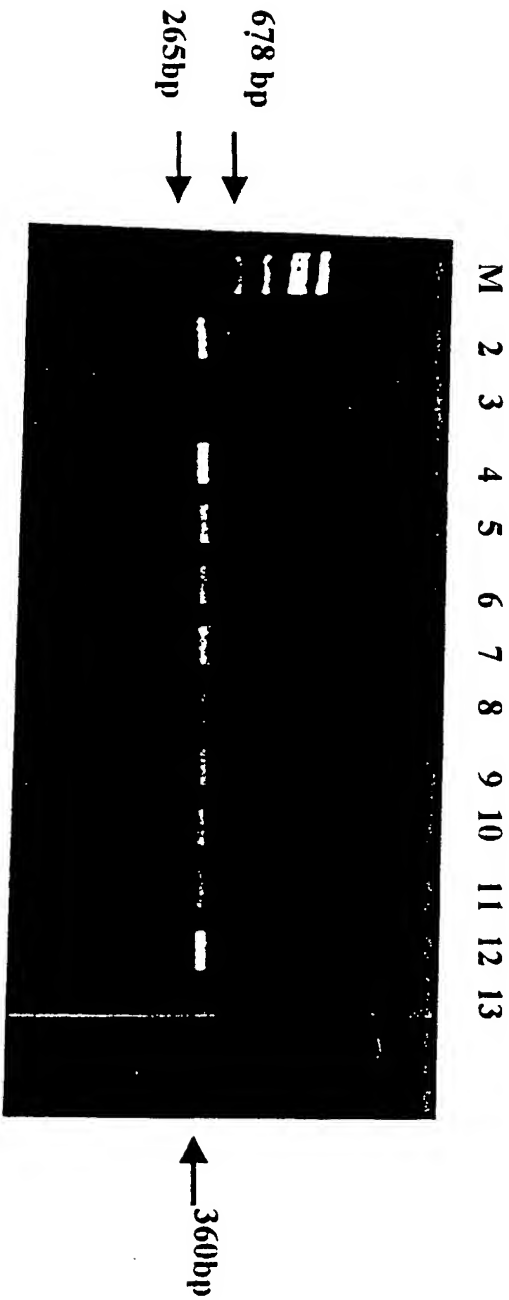


FIGURE 2

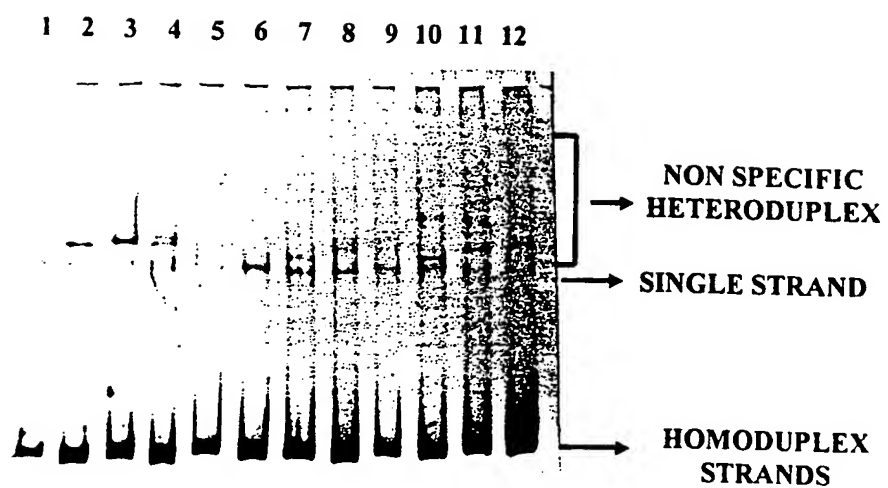


FIGURE 3

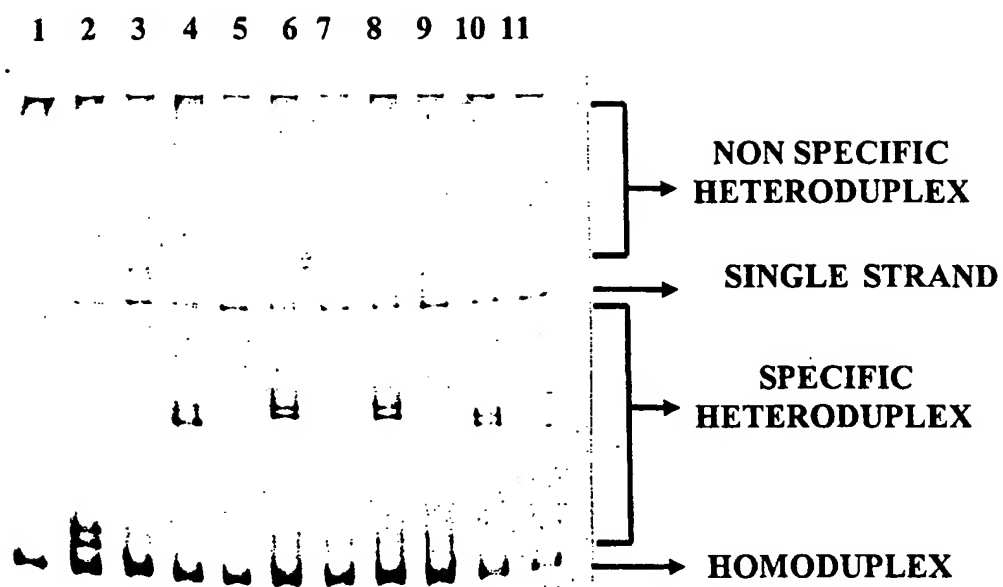


FIGURE 4

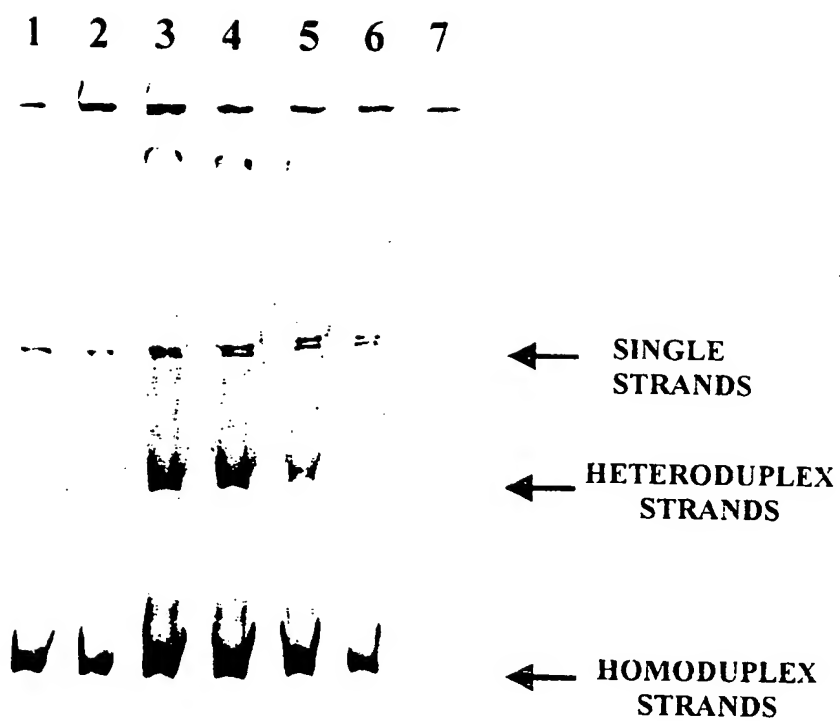




FIGURE 5

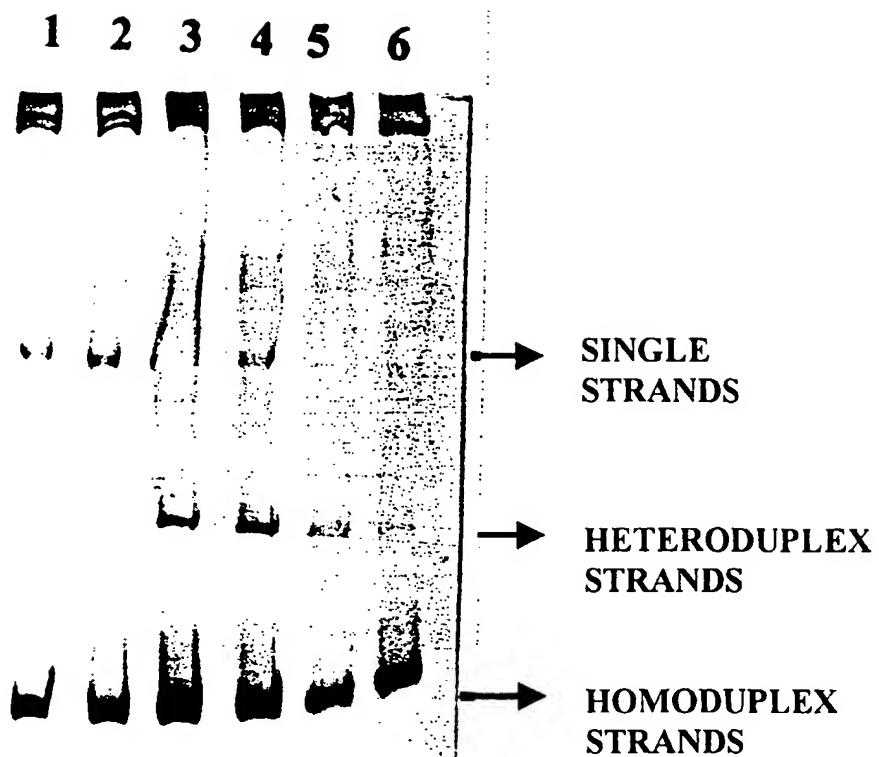
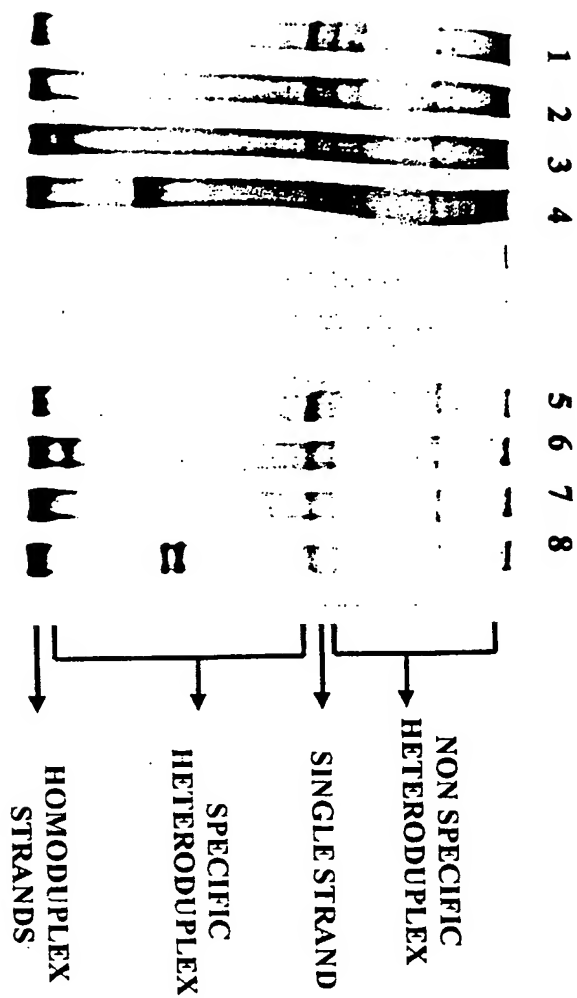
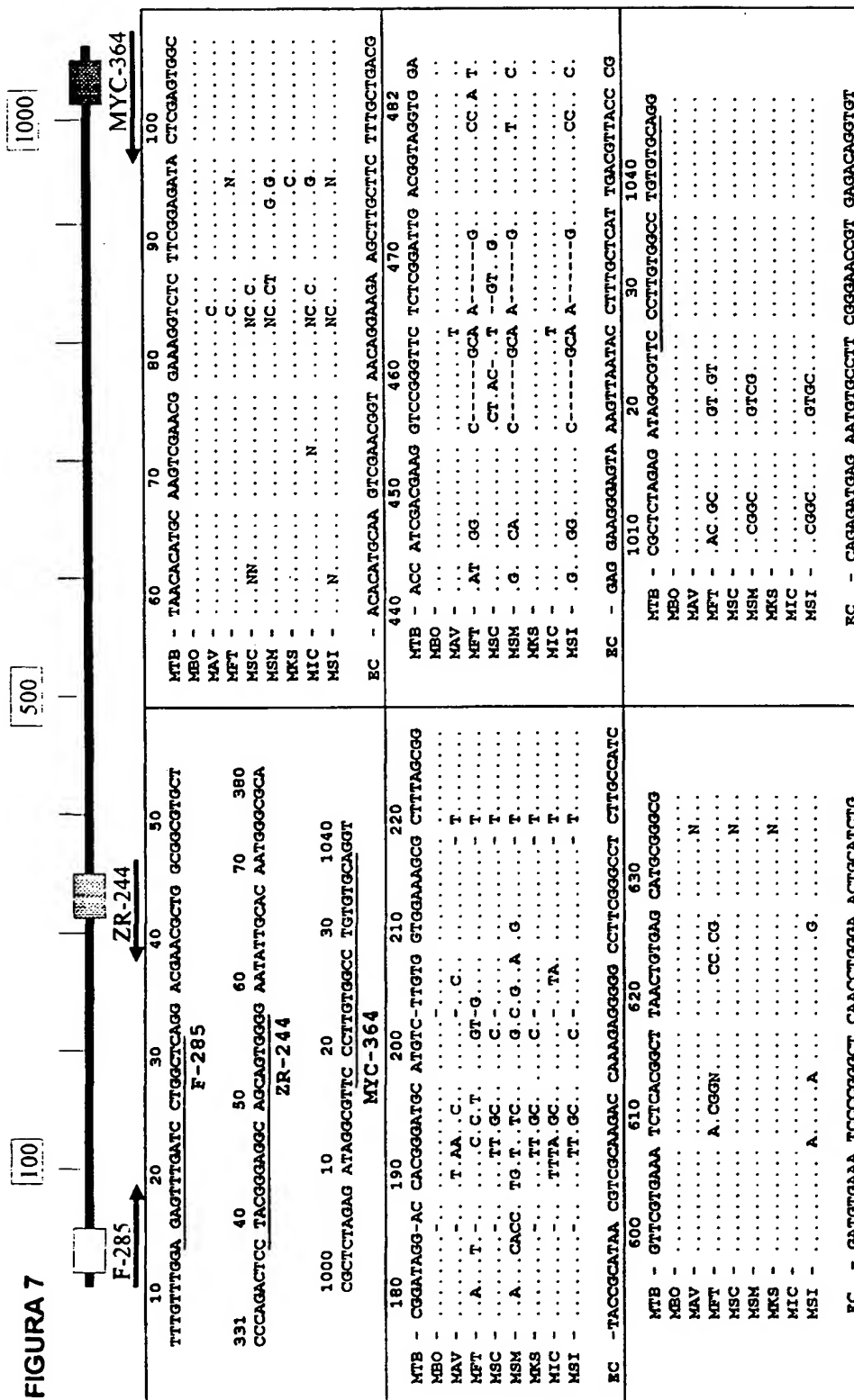


FIGURE 6



## FIGURA 7



MSQ= *N. serifulacum* MSN= *N. spumantis*  
E. coli

MTB= *M. tuberculosis* MBO= *M. bovis* MAV= *M. avium* MFT= *M. fortuitum*  
MKS= *M. kansasii* MNC= *M. mageritensis* MSI= *M. simium* ECT= *M. terrae*

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/BR 97/00087

A. CLASSIFICATION OF SUBJECT MATTER		
IPC <sup>6</sup> : C 12 Q 1/68		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED		
Minimum documentation searched (classification system followed by classification symbols)		
IPC <sup>6</sup> : C 12 Q 1/68		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)		
WPI, EPODOC		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 97/16 564 A1 (CIBA CORNING DIAGNOSTICS CORP.) 09 May 1997 (09.05.97), claim 14.	1
A	US 5 652 106 A (PLIKAYTIS et al.) 29 July 1997 (29.07.97), abstract.	1
A	Patent Abstracts of Japan, Vol.15, No.403 (C-875), 1991, JP 3-164199 A (SHIMA KENKYUSHO K.K.) 16 July 1991 (16.07.91), abstract.	1
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<input type="checkbox"/> Further documents are listed in the continuation of Box C. <input checked="" type="checkbox"/> See patent family annex.		
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "G" document member of the same patent family		
Date of the actual completion of the international search		Date of mailing of the international search report
26 August 1998 (26.08.98)		08 September 1998 (08.09.98)
Name and mailing address of the ISA: Austrian Patent Office Kohlmarkt 8-10; A-1014 Vienna Facsimile No. 1/53424/535		Authorized officer Wolf Telephone No 1/53424/436

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/BR 97/00087

In Recherchenbericht angeführtes Patentedokument Patent document cited in search report Document de brevet cité dans le rapport de recherche	Datum der Veröffentlichung Publication date Date de publication	Mitglied(er) der Patentfamilie Patent family member(s) Membre(s) de la famille de brevets	Datum der Veröffentlichung Publication date Date de publication
WO A1 9716564	09-05-97	AU A1 73268/96 EP A1 858514 US A 5731150	22-05-97 19-08-98 24-03-98
US A 5652106	29-07-97	keine - none - rien	